Yeast surface display for screening combinatorial polypeptide libraries

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Display on the yeast cell wall is well suited for engineering mammalian cell-surface and secreted proteins (e.g., antibodies, receptors, cytokines) that require endoplasmic reticulum-specific post-translational processing for efficient folding and activity. C-terminal fusion to the Aga2p mating adhesion receptor of *Saccharomyces cerevisiae* has been used for the selection of scFv antibody fragments with threefold decreased antigen dissociation rate from a randomly mutated library. A eukaryotic host should alleviate expression biases present in bacterially propagated combinatorial libraries. Quantitative flow cytometric analysis enables fine discrimination of kinetic parameters for protein binding to soluble ligands.

Keywords: antibody engineering, combinatorial library, surface display, affinity maturation, scFv

In the absence of quantitative computational structure-function relationships for proteins, rational approaches to mutagenesis have limited potential for success in rapidly altering protein molecular properties to meet predefined criteria. An alternative strategy, directed evolution through random mutagenesis and selection from combinatorial libraries, has yielded numerous successes. In particular, improvement of binding properties of recombinant antibodies through in vitro affinity maturation by phage display has revolutionized the field of antibody engineering—a field in which progress has generally depended upon methodologic advances. Phage display refers to genetic fusion of the peptide or protein of interest to a coat protein, typically pIII, of filamentous phage[1]. Phage libraries are screened by "panning" the displaying particles against immobilized antigen. Phage display technology[2-4] concomitantly selects the phenotype (antigen-binding activity) and the DNA encoding it. We describe an analogous library-screening system using the baker's yeast *Saccharomyces cerevisiae* as the displaying particle.

Phage-displayed libraries of in vitro recombined antibody variable (V) genes have enabled the generation of novel human antibodies with specificities directed against numerous antigens, including several human autoself specificities[2-4]. The ability to produce monoclonal human antibodies is significant for cancer immunotherapy, because human antibodies are less likely to induce a neutralizing antibody response from the patient's immune system. Therapeutic efficacy of antitumor radioimmunoconjugates has been shown experimentally to increase with antibody affinity[5], but the affinities of antibodies generated from phage displayed V gene libraries are not generally high enough for effective use in tumor therapy[6]. Thus, human antibodies isolated from phage libraries must be affinity matured to obtain tighter binding mutants. Phage-display has been successfully used in this capacity[7-8]; however, very-high-affinity antibodies (e.g., $K_d < 0.1$ nM) produced through affinity maturation by phage display have proven difficult to achieve.

A significant limitation of affinity maturation by phage display is the unpredictable expression bias against some eukaryotic proteins expressed in *Escherichia coli*, because incorporation of any protein fusion into the phage particle depends upon the ability of *E. coli* to express that protein in soluble form. *E. coli* possesses a limited ability to solubly express many disulfide-bonded mammalian proteins such as antibodies, because it lacks foldases and chaperones present in the endoplasmic reticulum that are required for efficient folding. Even closely related sequences can demonstrate widely different expression characteristics in *E. coli*; single amino acid changes within the CDRs of Fab fragments often completely eliminate expression[11], and random sampling of a single-chain Fv antibody fragment (scFv) phage library indicates that half of the library sequences lead to no detectable level of scFv in the culture supernatant[12]. Phage display may also select for properties other than increased affinity, such as reduced host toxicity, increased phage-particle infectivity, or dimerization to increase avidity[4,13]. Elution of particularly high-affinity phage clones from immobilized antigen can also be problematic, requiring severe conditions of low pH and high concentrations of chaotropic salts, which in turn can reduce phage infectivity.

Antibodies have also been displayed on the surface of *E. coli* using several different fusion systems to target the sequences of interest to the outer membrane[14,15]. Cells displaying an anti-digoxin scFv antibody were enriched from control cells by flow cytometric cell sorting[16]. These *E. coli* display technologies present several potential advantages with respect to phage display. A single phage particle displays one to five copies of a polypeptide sequence fused to pIII while *E. coli* can display thousands, avoiding stochastic fluctuation effects. In addition, screening by flow cytometry allows finer affinity discrimination compared with panning on immobilized antigen, which has been found to yield as little as 3.6×10^{-6}% recovery of input phage from a single selection step[17]. However, like phage display, affinity maturation of antibodies by *E. coli* display is limited by potential library bias due to expression of library members in a prokaryotic host poorly adapted for post-translational processing of mammalian proteins. Additionally, steric interference with the lipopolysaccharide layer of *E. coli* may impede binding to large macromolecular antigens such as proteins.

In contrast to *E. coli*, the yeast *S. cerevisiae* possesses protein folding and secretory machinery strikingly homologous to that of mammalian cells. Yeast, as an easily cultured single-cell microbe with facile genetics, is better suited for library methods than cultured mammalian cells (exemplified by the extensive use of the yeast two-hybrid system to study protein interactions[18]). Thus, a eukaryotic display system using *S. cerevisiae* as the host organism should alleviate library biases towards soluble expression in *E. coli* while retaining the benefits of large numbers of displayed fusions

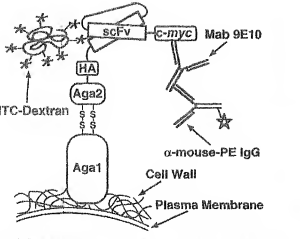

Figure 1. Schematic illustration of surface display on yeast. A nine-amino acid peptide epitope from the hemagglutinin antigen (HA) was fused to the C-terminus of the Aga2p subunit of *a*-agglutinin, followed by the 4-4-20 antifluorescein scFv sequence. An additional 10-residue epitope tag (*c-myc*) was fused at the C-terminus of the scFv, allowing quantitation of fusion display independent of antigen binding by either the HA or *c-myc* tags.

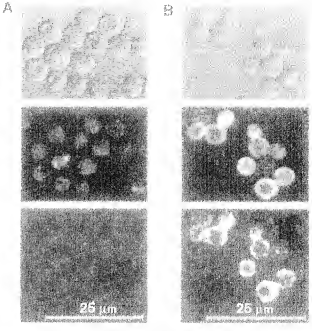

Figure 2. Confocal microscopic images of yeast displaying scFv. Yeast containing a plasmid directing surface expression of the HA peptide (A) or the scFv fusion (B) were labeled with Mab 9E10, followed by a secondary antimouse IgG-R-phycoerythrin (PE) conjugate and FITC-dextran. DIC (upper panels), red PE fluorescence (middle panels), and green FITC fluorescence (lower panels) images were collected.

per cell and flow cytometric screening capable of precise quantitative discrimination. Yeast adhesion receptors were used as a surface display scaffold to isolate 4-4-20 antifluorescein scFv mutants with slowed kinetics of dissociation from antigen.

Results and discussion

Development of display scaffold. Yeast possesses two related cell-surface receptors known as *a*-agglutinin and α-agglutinin that function to mediate cell-cell adhesion between *a* and α haploid cells as a prelude to fusion to form the diploid[16]. α-agglutinin has been shown to be linked covalently to cell wall glucan by the C-terminus[16,17], and *a*-agglutinin is believed to be anchored by a similar linkage[16]. Fusion to the C-terminal portion of α-agglutinin has been used to anchor enzymes and viral antigens on the yeast surface[21].

As a model system for development of the yeast surface display library screening method, we displayed a functional antifluorescein scFv and c-*myc* epitope tag on the cell wall of yeast by fusion to *a*-agglutinin, which unlike α-agglutinin is a two-subunit glycoprotein (Fig. 1). The 725 residue Aga1p subunit anchors the assembly to the cell wall[22] via β-glucan covalent linkage[16]; the 69-amino acid binding subunit Aga2p is linked to Aga1p by two disulfide bonds[23]. The native *a*-agglutinin binding activity is localized to the C-terminus of Aga2p[23]; thus, this represents a molecular domain with accessibility to extracellular macromolecules and a useful site for tethering proteins for display. We have constructed a vector for displaying proteins as C-terminal fusions to Aga2p (Fig. 1).

Verification of expression and surface localization of scFv. Expression of the Aga2p-scFv fusion is directed by the inducible GAL1 promoter[24]. Growth of yeast on glucose medium allows essentially complete repression of transcription from the GAL1 promoter, an important consideration for avoiding counterselection against sequences that negatively influence host growth. Switching cells to medium containing galactose induces production of the Aga1p and Aga2p fusion gene products, which associate within the secretory pathway and are exported to the cell surface. Surface localization of the Aga2p-scFv fusion has been verified by confocal fluorescence microscopy and flow cytometry. Cells labeled simultaneously with an anti–c-*myc* monoclonal antibody (Mab) and fluorescein-conjugated dextran (FITC-dextran) were examined by laser scanning confocal microscopy (Fig. 2). Control cells bearing a vector that directs display of an irrelevant peptide (i.e., a hemagglutinin, HA, epitope tag only) are not labeled by Mab specific for the *c-myc* epitope or FITC-dextran (Fig. 2A). In contrast, cells bearing the surface display vector pCT202 expressing the Aga2p-scFv–*c-myc* fusion are colabeled by both the anti–*c-myc* antibody and FITC-dextran (Fig. 2B), demonstrating that the antigen-binding site is accessible to very large macromolecules. Both of these strains are positively stained by Mab 12CA5 directed against the HA epitope tag (data not shown). Accessibility of the fusion for binding to both an intact IgG (150 kDa) and a 2×10^4 Da dextran polymer indicates an absence of significant steric hindrance from cell-wall components, a significant advantage relative to *E. coli* surface-displayed proteins, which are buried within a lipopolysaccharide layer that forms a barrier to macromolecular diffusion.

Two-color flow cytometric analysis of these yeast strains likewise demonstrates accessibly displayed scFv on the cell surface. Negative control and scFv-displaying strains were labeled with the anti–*c-myc* Mab 9E10 and FITC-dextran simultaneously. Bivariate histograms demonstrate a linear relationship between the intensity of phycoerythrin (PE) fluorescence (level of Mab 9E10 binding) and FITC fluorescence (antigen binding) for the cell population carrying the 4-4-20 display plasmid, while the control population exhibits background fluorescence (Figs. 3A and B). The distribution of fluorescence intensity within the positive fraction illustrates the importance of correcting the antigen-binding signal for cell-to-cell variability in the number of displayed fusions, as determined by epitope tag labeling. Quantitation of the display efficiency by comparison of an scFv-displaying cell population with calibration standards of known antibody binding capacities yields an average value of greater than 3×10^4 fusions per cell. Treatment of cells displaying the Aga2p-scFv fusion with dithiothreitol prior to labeling eliminated staining of the cell surface by both FITC-dextran and

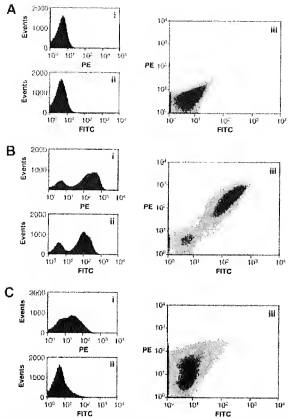

Figure 3. Flow cytometric analyses of yeast displaying scFv. Yeast strains displaying either (A) an irrelevant peptide or (B) the 4-4-20 scFv were labeled with Mab 9E10 and FITC-dextran. Cells displaying scFv were also treated with 5 mM DTT prior to labeling (C). (i) Univariate histograms of PE fluorescence associated with labeling by 9E10; (ii) univariate histograms of FITC fluorescence; (iii) bivariate histograms showing correlation between PE and FITC fluorescence.

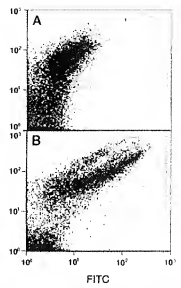

Figure 4. Enrichment of yeast displaying improved scFv variants by kinetic selection and flow cytometric cell sorting. Yeast expressing a mutagenized 4-4-20 scFv library (A) and a yeast pool resulting from three rounds of kinetic selection and amplification (B) were subjected to competitive disaociation of fluorescent antigen with 5-aminofluorescein, leaving cells displaying the tightest binding mutants with the highest ratio of FITC intensity/PE intensity.

Mab 9E10 (Fig. 3C), consistent with adherence of the fusion protein to the cell surface by a specific disulfide bonding interaction between the recombinant Aga2p subunit and Aga1p. This property illustrates another important feature of the yeast display system: Proteins can be simply released from the cell surface by reduction for further characterization.

To further examine the specificity of the 4-4-20/fluorescein interaction, a competitive dissociation assay was performed using a non-fluorescent analog of fluorescein, 5-aminofluorescein (data not shown). Analysis of these data yielded a monovalent dissociation rate constant (k_{off}) at 21°C of 3.7×10^{-3}/sec for FITC-dextran, and 3.9×10^{-3}/sec for fluorescein-biotin. Extrapolation of the exponential fit to $t = 0$ sec shows that the average valency of the interaction of an FITC-dextran molecule with scFv is less than 1.5. Similar results were obtained using fluoresceinated inulin, fluorescein-conjugated bovine serum albumin, and fluorescein-biotin as the competitor, indicating that the labeling of cells by FITC-dextran or fluorescein-biotin is due to a specific interaction between the displayed fusion and the fluorescein moiety. Furthermore, dissociation kinetics of fluorescein disodium salt (FDS) from surface displayed 4-4-20 scFv matched those from yeast-produced soluble 4-4-20 scFv as observed by spectrofluorometry (data not shown).

Enrichment of displaying cells by flow cytometric cell sorting. To determine the effectiveness of flow cytometric sorting with yeast surface display, mixtures of yeast bearing the surface display vector with those lacking the associated selectable marker were sorted and purities independently determined by replica plating. Significant enrichment factors (up to 600-fold) are obtained (Table 1). Thus, rare clones may be selected from yeast-displayed libraries by initially enriching positive cells at relaxed stringency and high yield to provide a smaller population, which can then be subjected to several passes of more stringent sorting to isolate rare clones.

Isolation of mutant scFv with lower koff from a yeast displayed mutagenized library. Selection of scFv genes randomly mutagenized by propagation in a "mutator" strain of *E. coli* has been described[29]. A library of approximately 5×10^5 4-4-20 scFv mutants created by propagation of the yeast surface-display vector in such a strain was expressed in yeast. The pool of cells displaying the scFv library were subjected to kinetic selection by competition of FITC-dextran labeled cells with 5-amiuofluorescein. c-*myc* positive cells exhibiting the highest ratio of FITC to PE fluorescence were collected by flow cytometric sorting (Fig. 4A), amplified by regrowth under fusion-repressing conditions (glucose carbon source), induced for surface fusion display, and resorted. Cells demonstrating a substantially increased persistence time of labeling by FITC-dextran were dramatically enriched following three rounds of sorting and amplification (Fig. 4).

FITC-dextran dissociation kinetics for two individual clones selected from the scFv library differed by 2.9-fold compared with wild-type 4-4-20 scFv (Fig. 5). Rate constants for the mutants were 1.9×10^{-3}/sec (mutant 4M1.1) and 2.0×10^{-3}/sec (4M1.2) at 23°C, compared with 5.6×10^{-3}/sec for wild-type; similar experi-

Table 1. Sorting enrichments of scFv-displaying yeast.

Initial purity	Sorted purity	Enrichment ratio
14%	83%	6.0
0.5%	95%	200
0.1%	59%	600

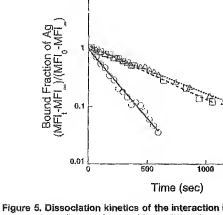

Figure 5. Dissociation kinetics of the interaction between fluorescein and surface displayed scFv. Yeast displaying 4-4-20 scFv (circles), mutant 4M1.1 (squares) isolated from the library, and mutant 4M1.2 (triangles) were labeled with Mab 9E10 and FITC-dextran, 5-aminofluorescein was added as a competitor. Mean intensity of FITC fluorescence of the 9E10 positive population of cells was followed as a function of time. The slope of the line is equal to the kinetic dissociation rate, and the extrapolated value at time t = 0 sec is equal to the valency of the interaction. MFI, = relative mean fluorescence intensity of yeast at time t = i.

ments yielded k_{off} values for fluorescein-biotin of 2.4×10^{-3}/sec, 2.8×10^{-3}/sec, and 5.0×10^{-4}/sec, respectively (data not shown). Additionally, soluble fluorescein dissociation kinetics determined by spectrofluorometry demonstrated a 2.2-fold improvement for both mutants relative to wild-type, and initial equilibrium fluorescence quenching experiments suggest a similar improvement in the affinity constant of the binding reaction (data not shown). Isolation of clones with only threefold reduced off-rate demonstrates the ability of this screening method to achieve precise quantitative distinctions.

Of 26 selected clones individually analyzed, two were identically improved in k_{off} (4M1.1 and 4M1.2, described above); two demonstrated wild-type k_{off} with a decrease in *c-myc* labeling skewing the linear expression level/activity relationship; one exhibited wild-type k_{off} and *c-myc* labeling; and 21 bound with an apparent k_{off} approximately 10-fold lower than wild-type only to polyvalent 2×10^6 Da FITC-dextran, but not to monovalent FITC-dextran or fluorescein-biotin (data not shown). Enrichment for clones with increased avidity resulted from use of polyvalent antigen (approximately 90 fluoresceins per dextran); avidity effects can be effectively avoided by appropriate design of screening conditions to ensure monovalent antigen binding. Furthermore, selection of epitope tag mutants can be eliminated by alternately detecting expression level by *c-myc* and HA tag labeling in sequential sorting rounds, or by alternative mutagenesis strategies targeting changes only to the scFv gene.

These results show that scFv fragments can be displayed on the surface of yeast in a manner accessible for macromolecular recognition and amenable to combinatorial library construction and screening. The displayed scFv specifically binds antigen—the first demonstration of a functional antibody fragment displayed on the yeast cell surface. The application of this display system to library methods for in vitro antibody affinity maturation and for display of other mammalian proteins is a significant complementary alternative to existing technologies such as phage display, bacterial surface display, and the yeast two-hybrid method. Indeed, the literal first-attempt success of the yeast display system in recovery of improved fluorescein-binding scFv mutants from a relatively small library under nonoptimized screening conditions clearly demonstrates the robustness of this technology. The demonstrated highly quantitative kinetic analysis of surface-tethered scFv and fine discrimination of clones with similar binding characteristics further attests to the great potential of yeast display for combinatorial optimization of proteins.

Experimental protocol

Construction of vectors for surface fusion. The AGA1 and AGA2 open reading frames were cloned by PCR from a yeast genomic library (CEN BANK, American Type Culture Collection, Rockville, MD). The 350-bp AGA2 PCR product was ligated into the cloning vector pCR-Script (Stratagene, La Jolla, CA) following the manufacturer's protocol. An oligonucleotide cassette encoding a nine-residue epitope tag (HA) was constructed by annealing complementary oligonucleotides, and this cassette was ligated into the pCR-Script-AGA2 vector. The resulting AGA2-HA open reading frame was subsequently subcloned into yeast shuttle vector pYC-G-BPTI (ref. 26). The inducible GAL1 promoter[25] was inserted upstream of the AGA2 open reading frame. The α1 mating factor transcriptional terminator was inserted following AGA2 by adapting its flanking restriction enzyme sites to XhoI and SacI and excising from vector pYC-G-BPTI, creating vector pCT201. The 2.2-kbp AGA1 PCR product was cloned into integrating vector YIplac211 (ref. 27) to create vector pIU211. The antifluorescein scFv 4-4-20 was likewise cloned by PCR and the pCR-Script kit. The 3′ primers for PCR contained a flanking nucleotide sequence encoding a 10-amino acid *c-myc* peptide epitope. An 813-bp NheI/XhoI fragment was excised from pCR-Script and subsequently subcloned into surface display vector pCT201 as an in-frame fusion to the 3′ end of AGA2, creating vector pCT202. Two epitope tags (HA and *c-myc*) were incorporated into the fusion to allow assessment of fusion display decoupled from fluorescein-binding activity. Vector pCT302 was created by inserting a synthetic oligonucleotide (UIUC Biotechnology Center) encoding a $(Gly_4\text{-}Ser)_3$ linker in frame between the AGA2 and 4-4-20 open reading frames of pCT202. Vector pIU211 was digested at a unique BstWI site within the AGA1 open reading frame and transformed into *S. cerevisiae* strain BJ5465 (*α ura3-52 trp1 leu2Δ1 his3Δ.200 pep4::HIS2 prb1Δ1.6R can1 GAL*) (Yeast Genetic Stock Center, Berkeley, CA) along with pCT201 or pCT202 by electroporation using a Bio-Rad (Richmond, CA) Gene Pulser Transfection Apparatus; the linearized vector was stably integrated at the native chromosomal AGA1 locus by homologous recombination, creating strain EBY100 and allowing expression of the Aga1p protein subunit from a promoter identical to that of the Aga2p/4-4-20 fusion protein.

Fluorescent labeling of yeast. Cells from 3 ml cultures in exponential phase (OD_{600} = 0.5–1.5) were harvested by centrifugation, washed with TBS (10 mM Tris-HCl, pH 7.5, 140 mM NaCl, 5 mM EDTA), and suspended in 100 μl TBS plus Mab 9E10 (1:100 dilution of raw ascites fluid; Berkeley Antibody Co., Richmond, CA) and/or 10 μM FITC-dextran (molecular weight 2×10^6, 0.008 mol FITC/mol glucose; Sigma, St. Louis, MO). Primary incubations were 30 to 60 min at room temperature. Cells were then pelleted and washed with TBS before resuspending in TBS plus R-PE-conjugated goat antimouse IgG (Sigma) and/or 10 μM FITC-dextran and incubating 20 to 30 min on ice. Cells were then pelleted and washed once with ice-cold TBS, followed by resuspension in ice-cold TBS to a density of approximately 5×10^7/ml for microscopy, or 2×10^8 to 1×10^9/ml for flow cytometry. For experiments using biotin-fluorescein, cells were grown, induced, harvested, and labeled as described above with 10 μM biotin-fluorescein in place of FITC-dextran as the primary label, and a mixture of 3 μg of streptavidin-PE and 1 μg of RED613-conjugated goat antimouse $F(ab')_2$ (Life Technologies, Grand Island, NY) as the secondary labeling reagents.

Confocal fluorescence microscopy. Yeast containing plasmid-directing surface expression of the HA peptide (pCT201) or the scFv fusion (pCT202) were grown for 20 h in medium containing 2% galactose as the only carbon source and subsequently labeled with Mab 9E10, followed by a secondary antimouse PE conjugate and FITC-dextran, as described. The labeled cells were mounted on polylysine-coated slides in 90% glycerol mounting medium containing 1 mg/ml *p*-phenylenediamine as an antibleaching reagent and analyzed with a laser scanning confocal microscope (UIUC Beckman Institute Microscopy Suite) at a rate of 8 sec with a 63x power objective. Images from DIC, red PE fluorescence, and green FITC fluorescence were collected.

Flow cytometric analysis and sorting. Labeled yeast cell suspensions were analyzed on a Coulter Epics XL flow cytometer at the Flow Cytometry Center of the UIUC Biotechnology Center. Event rate was maintained near 500 cells/sec. The population was gated by light scatter to avoid examination

of clumped cells, and data for 100,000 events were collected. For initial cell sorting experiments, yeast carrying the pCT202 vector were mixed with the untransformed parent strain BJ5465 and sorted based on FITC signal on a Coulter 753 cell sorting bench modified with CICERO sorting electronics (UIUUC Flow Cytometry Center). Presorted and sorted samples were plated on nonselective medium, then replica plated onto medium selective for the pCT202 vector. Purity was determined as the fraction of nonselective colonies that were viable on selective plates.

Quantitation of surface antibody expression level. Cells bearing vector pCT202 and Quantum Simply Cellular beads (Sigma) were labeled with FITC-conjugated Mab 12CA5 (Boehringer Mannheim, Indianapolis, IN) at 10 μg/ml in TBS as described and analyzed on a Coulter Epics XL flow cytometer. Comparison of the fluorescence intensity of the yeast sample with the standard beads allowed determination of antibody-binding capacity of the displaying yeast cells by linear regression using QuickCal for Quantum Simply Cellular (Sigma).

Kinetic analysis of antigen dissociation from cells displaying scFv. Yeast cells bearing plasmid pCT202 were grown and labeled with anti-*c-myc* Mab 9E10 and FITC-dextran or biotin-fluorescein, as described. A fraction of the labeled population was analyzed flow cytometrically to determine the initial level of fluorescence. Nonfluorescent competitor (5-aminofluorescein) was added to a final concentration of approximately 10 μM (approximately 1,000-fold excess) and the FITC or PE fluorescence of the *c-myc*–positive cell population was followed as a function of time at room temperature (21°C to 23°C) on a Coulter Epics XL. Data were fitted as an exponential decay. The probability that a polyvalent antigen is bound to the cell as a function of time is given by $P = 1 - (1 - e^{-kt})^n$, where N is the valency, *k* is the kinetic rate constant for dissociation, and t is time. For long times t, this reduces to $P = Ne^{-kt}$. Thus, extrapolation of data for long t to time zero yields $P = N$, or a fluorescence intensity of $F_{co} = N F_{ss}$ where F_{ss} is the extrapolated fluorescence at the time of competitor addition and F_o is the actual initial fluorescence. The valency of the interaction of surface displayed scFv 4-4-20 and polyvalent FITC-dextran was therefore determined as the y-intercept of the curves in Figure 5.

Binding to soluble fluorescein (FDS) was assayed by observing fluorescence quenching by whole cells displaying scFv. Cells were suspended at 2×10^7 cells/ml in TBS + 0.1% BSA in a quartz cuvette thermostatted at 23°C and titrated with FDS over a range of 0 to 7.5 nM. Fluorescence at 520 nm was observed with an SLM Aminco SPF-500 spectrofluorometer using 488 nm excitation. Control cells displaying an irrelevant scFv were titrated to obtain a slope for a two-parameter fit of an equilibrium-binding model to the data, yielding equilibrium constants and effective scFv concentrations. Following the equilibrium titration, 5-aminofluorescein was added to 1 μM and the change in fluorescence of the sample followed with time to determine k_{off} for FDS.

Mutagenesis of scFv gene. Approximately 100 ng of pCT302 were transformed in duplicate into *E. coli* strain XL1-Red (Stratagene) according to the manufacturer's protocol. Following 1-h induction in SOC medium, the two transformant groups were pooled and 1/2000 of the pooled plated on LB medium containing 100 μg/ml ampicillin to determine transformation efficiency. Five milliliters of liquid LB medium containing 50 μg/ml ampicillin plus 100 μg/ml carbenicillin (LB-AMP50-CARB100) were inoculated with the remainder of the transformants and grown overnight at 37°C ($OD_{600} \approx 1.0$). A sufficient volume of this culture was collected to inoculate 50 ml LB-AMP50-CARB100 to $OD_{600} = 0.01$ in a baffled shake flask and grown to $OD_{600} \approx 1.0$ to 1.1 at 37°C. Cells were collected by centrifugation and used to inoculate 200 ml LB-AMP50-CARB100 to $OD_{600} = 0.001$, and the culture was grown at 37°C to $OD_{600} \approx 1.0$. Plasmid DNA was isolated by the Qiagen (Santa Clarita, CA) Maxiprep kit. The recovered DNA was retransformed into XL1-Red and the growth cycle repeated three times, yielding a final product subjected to approximately 90 generations of growth in the mutator strain.

Library expression and kinetic screen. Fifty micrograms of mutagenized pCT302 DNA were transformed into yeast strain EBY100 by the method of Gietz and Schiestl[9] in 10 separate reactions. The products were pooled, and 1/2000 of the total plated on selective medium to determine the total number of transformants. The remainder were inoculated into 50 ml of selective glucose medium, grown overnight at 30°C, passaged to $OD_{600} = 0.1$, and expanded tenfold. Selective galactose medium (5 ml) was inoculated to $OD_{600} = 0.5$ and grown overnight at 30°C to $OD_{600} \approx 1.0$ to 2.0. Samples of 10^7 cells (1 OD_{600}-ml) were labeled with FITC-dextran as described. Following labeling, cells were resuspended in 10 μM 5-aminofluorescein and 9E10 Mab at room temperature for 20 min, at which time samples were rinsed with ice-cold buffer to stop competitive dissociation of FITC-dextran and labeled with antimouse-PE secondary antibody as described above. Samples were sorted on a Coulter 753 bench with a sort window as shown in Figure 4 and with an event rate of 4,000/sec. During sorting round 1, 6×10^7 cells were examined and the window was set to collect 0.2% of the population. The collected cells were regrown in glucose medium and switched to galactose as described prior to repeating the competition and sorting. A total of four rounds of sorting and amplification were performed. In round 2, 4×10^7 cells were examined. In each of rounds 3 and 4, 2×10^7 cells were examined. Rounds 1 and 2 were performed in enrichment mode to provide a high recovery of all positive clones, and rounds 3 and 4 were performed in purify mode to reject coincident negative cells and achieve larger enrichment factors. The products of round 4 were plated to isolate individual clones.

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